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REMARKS

In response to the election of species requirement dated July 3, 2006, applicant elects chronic myeloid leukemia for examination, with traverse for the following reasons. The examiner relies on Nakamura et al., Molecular Immunology, 2000, which describes a humanized anti-GM2 monoclonal antibody. This anti-gm2 antibody is to treat $GM2^+$ cancers. In these cancers, the GM2 antigen is strongly expressed at the surface of the cells as shown in the enclosed Nakamura et al. Cancer Research, 1999, paper. "Gangliosides such as GD3, GM2 and GD2 are abundantly expressed at the cell surface of certain types of human cancer and have been shown to function as effective targets for passive immunotherapy with Mabs". See page 5323, left column. Therefore, the teaching of Nakamura et al, 2000, is the opposite of the present invention since the claims are directed to the treatment of pathologies for which the number of antigenic sites or the antigenic density is low.

Moreover, all of the restricted species can be examined without an undue burden.

IDS

An IDS was filed on August 9, 2000 citing 8 references cited by the International Searching Authority. The examiner is respectfully requested to return an initialed copy of the related Forms SB-08 with the next communication from the examiner.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check or credit card payment form being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to

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charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date October 6, 2006

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Apoptosis Induction of Human Lung Cancer Cell Line in Multicellular Heterospheroids with Humanized Antiganglioside GM2 Monoclonal Antibody

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ABSTRACT

The chimeric antiganglioside GM2 monoclonal antibody (MAb) KM966, which showed high effector functions such as complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC), potently suppressed growth and metastases of GM2-positive human cancer cells inoculated into mice. To further improve the therapeutic efficacy of the anti-GM2 MAb in humans, we constructed a humanized anti-GM2 MAb, KM8969. The humanized KM8969 was more efficient in supporting ADCC against GM2-positive human cancer cell lines than the chimeric KM966, whereas complement-dependent cytotoxicity was slightly reduced in the humanized KM8969. In addition, the humanized KM8969 was shown to exert potent ADCC mediated by both lymphocytes and monocytes. To investigate the effect of the humanized KM8969 on the biological function of GM2 in the condition physiologically mimicking formation and growth of cancer masses, the heterospheroids composed of normal human dermal fibroblasts and GM2-positive human lung cancer cells were developed. Interestingly, the humanized KM8969 gave rise to growth inhibition of heterospheroids without dependence of the effector functions. Morphological and immunocytochemical analysis suggested that the inhibitory effect was due to the apoptosis of GM2-positive cancer cells in the heterospheroids. The result indicates that GM2 captured by the antibody on the cell surface loses its physiological function that plays a critical role in maintaining the three-dimensional growth of cancer cells in contact with its own cells or other type of cells in a microenvironment. The humanized KM8969, which can destroy the cancer cells via blocking functional GM2 on the cell surface as well as the effector functions, would have extraordinary potential in human cancer therapy.

INTRODUCTION

Gangliosides, which constitute a class of cell membrane constituent glycolipids, are molecules composed of a carbohydrate chain with sialic acid at the cell surface and a hydrophobic ceramide in the lipid bilayers (1). It has been known that quantitative and qualitative changes occur in the expression of gangliosides through the oncogenic transformation of cells (2). Gangliosides such as GD3, GM2, and GD2[2] are abundantly expressed on the cell surface of certain types of human cancer and have been shown to function as effective targets for passive immunotherapy with MAbs[3] (3–14). Several anti-GM2 IgM antibodies were produced in mice, rats, and humans (14–19), and a human anti-GM2 IgM antibody showed some clinical responses in the treatment of melanoma (20). Moreover, in clinical studies, vaccination with GM2-keyhole limpet hemocyanin in melanoma patients resulted in the extension of disease-free intervals and survival when correlated with high serum titer anti-GM2 IgM and IgG antibodies (21, 22). In view of the foregoing, GM2 is expected to be an ideal antigen for specific immunotherapy of human cancers with either vaccination or MAbs.

We have previously reported the construction of a first mouse/human chimeric anti-GM2 MAb KM966, which consists of the constant (C) region of hIgG1-κ and the variable (V) region of the murine anti-GM2 IgM MAb KM696 (12). The chimeric KM966 seemed to be therapeutically effective in inhibiting the growth of human lung cancer cells implanted s.c. into nude mice and metastases formation of human lung cancer cells inoculated i.v. into NK cell-depleted SCID mice (12, 23). Chimeric MAbs, however, may have the possibility of inducing a substantial human antimouse antibody response because one-third of the molecule is still of murine origin (24). To further reduce the immunogenicity of chimeric MAbs and to prolong the circulating half-life, humanized MAbs are generated by grafting CDRs of murine MAbs into the backbone of human FRs and C regions (25, 26). Humanized MAbs contain about 10% murine origin residues and 90% human origin residues, and, thus, theoretically further reduction of the immunogenicity is achieved compared with chimeric MAbs.

In the present article, we constructed a humanized anti-GM2 MAb, KM8969, and studied its antigen-binding affinity and effector functions such as CDC and ADCC. ADCC is considered to be the major mechanism through which cancer cells, on treatment with anticancer MAbs, are eliminated *in vivo* (27, 28). The antimetastatic effect of the chimeric KM966 *in vivo* was mainly due to an ADCC reaction mediated by macrophages in the NK cell-depleted SCID mice (23). Furthermore, the chimeric KM966 was very effective in the lysis of human lung cancer cell lines mediated by both lymphocytes and monocytes (29). These facts suggest that one potentially important mechanism for the *in vivo* anticancer effects of the chimeric KM966 is its ability to mediate ADCC. Therefore, we also examined whether the humanized KM8969 is effective in ADCC mediated by lymphocytes and monocytes against various human lung cancer cell lines including the cells with the characteristics of multidrug resistance or high metastasis.

Many studies have noted that gangliosides function as receptors and are involved in signal transduction and that some are involved in the process of cell adhesion (30–35). In the past, Bjerkvig *et al.* (36) reported that murine anti-GM2 IgM antibodies induced necrosis in spheroids consisting of human glioma cells, which express high levels of GM2; however, there were no studies to demonstrate reproducibility in different *in vitro* culture systems and its cytotoxic mechanism. Multicellular spheroids have been shown to represent tissues and organs in a model in which the biological and morphological proper-

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[2] Gangliosides have been designated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (53) and to the coding system of Svennerholm (1).

[3] The abbreviations used are: MAb, monoclonal antibody; SCID, severe combined immunodeficient; NK, natural killer; CDR, complementarity-determining region; FR, framework region; C, constant (region); V, variable (region); ADCC, antibody-dependent cellular cytotoxicity; PNIPAAm, poly-*N*-isopropyl acrylamide; ADM, Adriamycin; CDDP, cisplatin; CDC, complement-dependent cytotoxicity; EC_{50}, concentration for half-maximal cytolysis; PBMC, peripheral blood mononuclear cell; hIgG, human IgG; SCLC, small cell lung cancer.

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ties are maintained in conditions similar to those that exist *in vivo* (37–40). In this study, heterospheroids were successfully developed by using a collagen-conjugated thermo-responsive polymer, PNIPAAm, as a cell substratum (37, 38). This method enabled us to regulate the size and the cell composition of resultant heterospheroids and to evaluate the interaction of the cancer cells with other types of cells, including fibroblasts. We investigated, with this heterospheroid culture system, the biological effects of the humanized KM8969 on GM2-positive cancer cells in the heterospheroids composed of normal human dermal fibroblasts and human lung cancer cells.

MATERIALS AND METHODS

Cell Lines. The human SCLC SBC-3 and SBC-5 cells were kindly provided by Dr. S. Hiraki (Okayama University, Okayama, Japan). The human lung squamous cell carcinoma RERF-LC-AI cells were kindly provided by Dr. M. Akiyama (Radiation Effects Research Foundation, Hiroshima, Japan). The human SCLC H69 cells were obtained from the American Type Culture Collection (Rockville, MD). The human lung adenocarcinoma PC-14 cells were kindly provided by Dr. N. Saijo (National Cancer Institute, Tokyo, Japan). The human large cell lung cancer PC-13 cells and the human stomach adenocarcinoma MKN-28 cells were obtained from Immuno Biological Laboratory (Tokyo, Japan). Two drug-resistant sublines of SBC-3 cells were obtained by culturing the cells with gradually increasing concentrations of ADM or CDDP. After 6 months, cells that grew in 100ng/ml ADM and 400ng/ml CDDP were obtained and named SBC-3/ADM and SBC-3/CDDP, respectively (41). PC-14-PM4, a variant cell line of PC-14, with higher metastatic potential to the pleural cavity, was established by repeated *in vivo* selection (42). Cell cultures were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. Normal human dermal fibroblasts were obtained from Kurabo Industries (Osaka, Japan). Normal human dermal fibroblasts and heterospheroids were maintained in DMEM containing 10% fetal bovine serum, 20 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air.

Antibodies. The chimeric anti-GM2 MAb KM966 was developed in our laboratory (12). Polyclonal hIgG was obtained from Organ Teknika Corporation (West Chester, PA).[4]

Construction of Humanized Antibody. A detailed process of humanization of the murine anti-GM2 MAb KM696 was described previously.[4] Briefly, a series of humanized anti-GM2 MAb variants that had various mutations in the amino acid residues of their FRs were constructed based on the molecular modeling analysis of their antibody V regions, and their binding affinities to GM2 were evaluated using a transient expression system in COS-7 cells. As a result, one of the humanized MAb variants, KM8969, which revealed a high binding affinity comparable to the chimeric KM966, was selected as a candidate for humanized anti-GM2 MAb; its biological activities including cytotoxic effector functions were further characterized. Stable expression and purification of the humanized KM8969 were performed as reported previously (12). In the humanization, the IgG1-κ isotype was chosen because it is the preferred human isotype for supporting potent cytotoxic effector functions.

Flow Cytometric Analysis. For indirect immunofluorescence, the cancer cells (1×10^6) were incubated with purified MAb (50 μg/ml) at 4°C for 1 h. The cells were washed in PBS and then incubated in FITC-labeled protein A (Boehringer Mannheim, Mannheim, Germany) at 4°C for 1 h. The cells were washed in PBS and analyzed on a flow cytometer, EPICS Elite (Coulter, Hialeah, FL). Five $\times 10^3$ cells were acquired by list mode and gated by forward light scatter *versus* side light scatter, thereby excluding dead cells and debris. For quantitating expression levels of GM2 on the cancer cells surface, flow cytometric analysis was also done using the chimeric KM966 (29).

CDC Assay. A CDC assay was performed as reported previously (43). Briefly, the cancer cells (5×10^6 cells) were labeled with 3.7 MBq of $Na_2{}^{51}CrO_4$ (^{51}Cr) at 37°C for 1 h and kept for 30 min at 4°C to remove loosely bound ^{51}Cr after washing. Aliquots of the labeled cancer cells were distributed into 96-well U-bottomed plates (5×10^4 cells/50 μl) and incubated with serial dilutions of MAbs (50 μl) at room temperature for 30 min. After centrifugation, the supernatants were removed, and aliquots of the diluted human serum were distributed (150 μl) as a source of the complement. After a 1-h incubation at 37°C, the plate was centrifuged, and the radioactivity in the supernatants was measured using a gamma counter. The percentage of specific cytolysis was calculated from the counts of samples according to the formula:

$$\%\ \text{specific lysis} = \frac{E - S}{M - S} \times 100$$

where E is the experimental release (cpm in the supernatant from cancer cells incubated with antibody and complement), S is the spontaneous release (cpm in the supernatant from cancer cells incubated with medium alone), and M is the maximum release (cpm released from cancer cells lysed with 1 N HCl). To evaluate CDC, concentrations of MAbs required for EC_{50} were calculated.

ADCC Assay. An ADCC assay was performed by 4-h ^{51}Cr-release assay as reported previously (43). Briefly, aliquots of the ^{51}Cr-labeled cancer cells as described in the CDC assay were distributed into 96-well U-bottomed plates (1×10^4 cells/50 μl) and incubated with serial dilutions of MAbs (50 μl) in the presence of human effector cells (100 μl) at 37°C for 4 h. Human PBMCs, separated from a healthy donor's peripheral blood using Polymorphprep (Nycomed Pharma AS, Oslo, Norway) according to the manufacturer's instructions, provided the effector cells. After centrifugation, the radioactivity in the supernatants was measured using a gamma counter. The percentage of specific cytolysis was calculated in the same way as in the CDC assay. To evaluate ADCC, concentrations of MAbs required for EC_{50} were calculated. Moreover, to analyze the effector cell populations in the human PBMCs that were involved in the ADCC of the humanized KM8969 against various human lung cancer cell lines, highly purified lymphocytes (>99%) and monocytes (>99%) were separated by centrifugal elutriation from human PBMCs and were used as described previously (29).

Preparation of Heterospheroids. Heterospheroids were prepared according to the previously reported method (37, 38). A thermo-responsive polymer, PNIPAAm, was used. PNIPAAm was insoluble in water over the lower critical solution temperature (~30°C) and was reversibly soluble below the lower critical solution temperature. The substratum, with surface area of about 9.6 cm², for cell culture and cell detachment was prepared by coating a hydrophilic culture dish with a uniform mixture of PNIPAAm and type I collagen and was called type I substratum. The formation and maintenance of heterospheroids were carried out on a hydrophilic culture dish coated with 1% agarose (type II substratum). Normal human dermal fibroblasts were seeded on the prewarmed (37°C) type I substratum at an initial cell density of 4.0×10^5/2 ml. After 3 days of culture at 37°C, the fibroblasts proliferated to a confluent state. Then aliquots of 1 ml of prewarmed (37°C) human cancer cells suspensions were seeded on the confluent fibroblasts monolayer at a cell density of 5.0×10^5/ml After 60 min of coculture at 37°C, it was confirmed, using a phase-contrast microscope, that more than 90% of the seeded cancer cells attached to the fibroblasts monolayer. Then the culture dishes were transferred to an ambient temperature (~25°C) and allowed to stand for about 5 min. By this procedure, the cancer cells-attached fibroblasts monolayer was completely detached from the type I substratum as a self-supporting sheet. The detached cell sheet was gently transferred into a new dish containing chilled PBS using a wide pipette tip. This process was repeated and finally carried out using chilled culture medium instead of PBS. Then the rinsed cell sheet was transferred into prewarmed (37°C) culture medium on type II substratum by the same pipetting procedure (day 0). The heterospheroids formation and culture were carried out at 37°C on the type II substratum, and the culture medium was changed every other day from day 2.

Antibody Treatment of Heterospheroids. The heterospheroids ($n = 10$) were incubated with the humanized KM8969 from day 2 to day 14. The diameter of the heterospheroids cultured for 2 days ranged from 700 to 850 μm. The freshly prepared medium containing 20 μg/ml antibody was used for each medium exchange at days 2, 4, 6, 8, 10, and 12. Two control groups received either polyclonal hIgG or medium alone.

Construction of Humanized MAb with Tag. For detecting GM2 in the heterospheroids, we constructed a humanized anti-GM2 MAb with tag composed of FLAG peptide (DYKDDDDK) on the COOH-terminal of the heavy-chain C region. Briefly, the synthetic DNA-encoding FLAG peptide was fused in-frame to the 3'-end of the heavy-chain C region cDNA. Then the modified

[4] K. Nakamura, Y. Tanaka, I. Fujino, K. Shitara, and N. Hanai. Construction and characterization of a humanized anti-ganglioside GM2 monoclonal antibody, submitted for publication.

cDNA was subcloned into the expression vector of the humanized KM8969 to replace the native heavy-chain C region cDNA. Stable expression and purification of the tagged humanized KM8969 were performed as described previously (12). The purified tagged MAb was named KM8969FLAG.

Morphological and Immunocytochemical Analysis of Heterospheroids. Paraffin-embedded sections and frozen sections of 7- and 14-day-cultured heterospheroids were prepared as follows. For paraffin sections, the heterospheroids were fixed in a 10% formalin neutral buffer solution for 60 min at 4°C. They were dehydrated and embedded in paraffin wax. Sections were obtained by cutting around their center at a thickness of 4 μm, dewaxed and stained with H&E by standard procedures. For immunoperoxidase staining, dewaxed sections were immersed in methanol containing 0.3% H_2O_2 for 30 min to remove endogenous peroxidase activity. For frozen sections, the heterospheroids were snap-frozen with OCT compound in liquid nitrogen. Sections were cut at a thickness of 5 μm, air-dried for 5 min, and fixed in a 10% formalin neutral buffer solution for 15 min. For immunoperoxidase staining, sections were washed with PBS and immersed in methanol containing 0.3% H_2O_2 for 30 min to remove endogenous peroxidase activity.

For detecting GM2 in the heterospheroids, frozen sections of 14-day-cultured heterospheroids were incubated with 10 μg/ml of the KM8969FLAG at 37°C for 1 h. Bound KM8969FLAG was detected using biotinylated mouse anti-FLAG M2 MAb (Eastman Kodak, New Haven, CT), followed by incubation with fluorescein avidin D (Vector Laboratories, Burlingame, CA).

For detecting the humanized KM8969 in the heterospheroids, frozen sections of 7- and 14-day-cultured heterospheroids were incubated with horseradish peroxidase-labeled goat anti-hIgG (H&L) (American Qualex, San Clemente, CA) at 37°C for 1 h. Bound peroxidase was detected after incubation with 0.5 mg/ml DAB containing 0.01% H_2O_2 for 2 min. Sections were counterstained in hematoxylin and dehydrated before mounting.

Apoptosis on paraffin sections of 14-day-cultured heterospheroids was detected using the *In Situ* Cell Death Detection kit (POD, Boehringer Mannheim) as described by the manufacturer. Sections were counterstained in methyl green and dehydrated before mounting.

All of the sections were observed with phase-contrast microscope or fluorescent microscope.

RESULTS

Humanized MAb and Characterization of Its Binding Activity. The binding affinities of the purified chimeric KM966 and humanized KM8969 for GM2 were measured using GM2-binding ELISA (12). From the dose titration curves of ELISA, the concentrations of MAbs corresponding to the midpoint absorbance (EC_{50}) were 0.045 ± 0.004 and 0.036 ± 0.003 μg/ml for chimeric KM966 and humanized KM8969, respectively (data not shown). In the case of the humanized KM8969, seven residues of FRs in the variable heavy region and nine residues of FRs in the variable light region as well as the residues of each CDR of the murine MAb were transferred to human FRs to attain high binding affinity. To confirm whether the binding characteristics of parental MAb were preserved in the humanized KM8969, we performed flow cytometric analysis using GM2-positive human SCLC SBC-3 cells. As shown in Fig. 1, the humanized KM8969 bound to SBC-3 cells at a slightly higher rate than did the chimeric KM966. We also examined antigen-binding specificity by ganglioside-binding ELISA (12). The humanized KM8969 reacted strongly with *N*-acetyl-GM2 and *N*-glycolyl-GM2 but weakly with GD2 of 11 common gangliosides (GM1, *N*-acetyl-GM2, *N*-glycolyl-GM2, *N*-acetyl-GM3, *N*-glycolyl-GM3, GD1a, GD1b, GD2, GD3, GQ1b, GT1b), which was the same reactive pattern as that of the chimeric KM966 (data not shown).

CDC of Humanized KM8969. CDC of the purified humanized KM8969 against SBC-3 cells was evaluated in the presence of various concentrations of human serum as complement (5–15%; Fig. 2). Both humanized and chimeric MAb led to an enhancement of complement activation at a serum concentration of 5%, but the increase of serum concentrations had no effect on the enhancement of antibody-dependent specific cytolysis. At a serum concentration of 15%, the concentration of the humanized KM8969 required for half-maximal CDC (EC_{50}) was 4.41 ± 0.15 *versus* 1.02 ± 0.07 μg/ml for the chimeric KM966. The slight loss of CDC despite the potent binding affinity to GM2 may reflect the slightly different binding characteristics of the humanized KM8969 compared with the chimeric KM966.

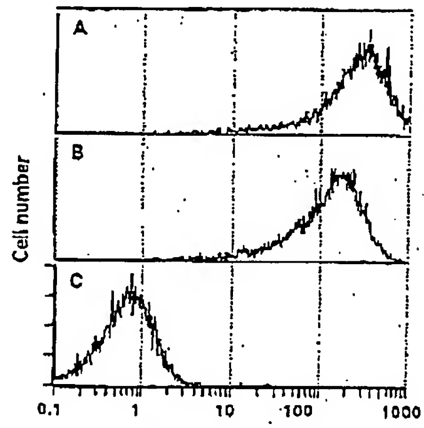

Fig. 1. Flow cytometric analysis of MAbs binding to SBC-3 cells. SBC-3 cells (1×10^6) were incubated with 50 μg/ml MAbs, and bound MAbs were detected with fluorescein-labeled protein A. *A*, humanized KM8969; *B*, chimeric KM966; *C*, control hIgG.

ADCC of Humanized KM8969. The ADCC of the purified humanized KM8969 against SBC-3 cells was evaluated in the presence of human PBMCs as effector cells at various E:T ratios (5:1, 10:1, and 20:1; Fig. 3). Both humanized and chimeric MAbs exhibited an enhanced cancer-cell killing even at an E:T ratio of 5:1, but the increase of E:T ratios seemed to have no effect on the enhancement of antibody-dependent specific cytolysis because of the high cytolytic activity of effector cells without MAbs. At an E:T ratio of 20:1, the EC_{50} were 0.17 ± 0.02 and 0.07 ± 0.02 μg/ml for chimeric KM966 and humanized KM8969, respectively. The humanized KM8969 exhibited an ADCC that was slightly higher than the chimeric KM966, presumably also reflecting different binding characteristics between humanized KM8969 and chimeric KM966. From the dose titration curves of ADCC, the optimal dose of the humanized KM8969 was determined at 1 μg/ml. To further analyze the ADCC of the humanized KM8969, lymphocytes and monocytes isolated from a healthy donor's PBMCs were each incubated with various human lung cancer cell lines in the presence of the optimal dose of MAb (1 μg/ml) and at an E:T ratio of 20:1. As shown in Table 1, the humanized KM8969 significantly induced ADCC mediated by both lymphocytes and monocytes against SBC-5 (SCLC), H69 (SCLC), PC-14 (lung adenocarcinoma), PC-13 (large cell lung cancer), and SBC-3 (SCLC) cells, but not RERF-LC-AI (lung squamous cell carcinoma) cells. The GM2 expression levels on the cancer cells surface were significantly correlated with the ADCC of the humanized KM8969 mediated by both lymphocytes and monocytes. The humanized KM8969 mediated a higher ADCC than the chimeric KM966 against GM2-positive human lung cancer cell lines, and the results supported the enhanced ADCC of the humanized KM8969 compared with the chimeric KM966, irrespective of the cell type. It was pointed out that the ADCC mediated by lymphocytes was higher than that mediated by monocytes.

We also evaluated ADCC against multidrug-resistant lung cancer cell lines and a highly metastatic lung cancer cell line (Table 2). The human-

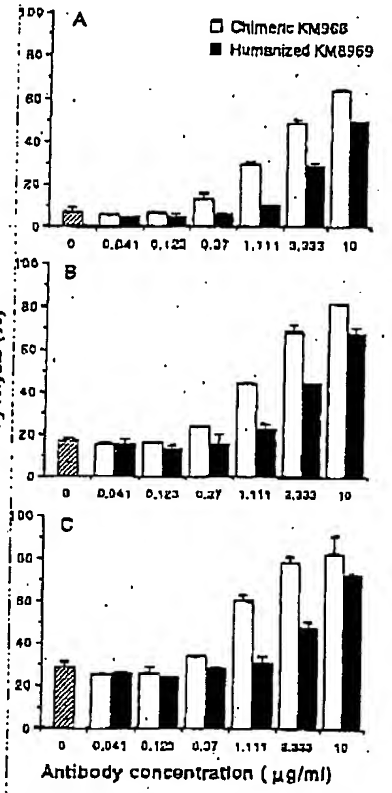

Fig. 2. CDC of MAbs against human lung cancer SBC-3 cells. ^{51}Cr-labeled SBC-3 cells were incubated at room temperature for 30 min with either chimeric KM966 (*open columns*) or humanized KM8969 (*closed columns*) at the concentrations indicated. After washing, diluted human serum with medium was added and incubated at 37°C for 1 h. Serum dilutions were 5% (*A*), 10% (*B*), or 15% (*C*). After centrifugation, the radioactivity in the supernatants was measured, and the percentages of cytolysis were plotted against the concentrations of MAbs. The lysis percentages observed with diluted serum alone (*striped columns*) were 6.7 ± 1.8, 16.8 ± 1.2, and 28.4 ± 2.6% for dilutions of 5, 10, and 15%, respectively.

ized KM8969 significantly induced ADCC mediated by lymphocytes and monocytes against two types of multidrug-resistant lung cancer cell lines, SBC-3/ADM and SBC-3/CDDP. Moreover, the humanized KM8969 exerted potent ADCC mediated by lymphocytes and monocytes against a highly metastatic cancer cell line, PC-14-PM4.

Morphology of Heterospheroids. The H&E staining of sections from central regions of 14-day-cultured heterospheroids are shown in Fig. 4. Fibroblasts and lung cancer SBC-3 cells were strictly localized in a heterospheroid. In the control heterospheroid with hIgG treatment, it was observed by high magnification that the thick rim cell layers of strongly H&E-stained viable cells fully covered the cells that were aggregated into masses within the heterospheroid (Fig. 4, *A* and *B*). The same result was obtained in another control heterospheroid with medium alone (data not shown). On the other hand, the rim cell layers of the heterospheroid treated with the humanized KM8969 were scarce (Fig. 4, *C* and *D*). These observations suggested that the humanized KM8969 caused growth inhibition of the cells in the rim layers of the heterospheroid.

Detection of GM2 in Heterospheroids. To detect GM2 in the heterospheroids, we constructed KM8969FLAG that had FLAG peptide on the COOH-terminus of the heavy chain. The KM8969FLAG retained binding affinity and specificity of the humanized KM8969 (data not shown). Immunofluorescence staining using the KM8969FLAG, biotinylated anti-FLAG M2 MAb, and fluorescein-avidin D enabled us to detect GM2 specifically with high sensitivity. As shown in Fig. 5*A*, the specific signals of GM2 that indicate the existence of GM2-positive SBC-3 cells were observed only in the rim layers of 14-day-cultured heterospheroids of the control group treated with hIgG. The same result was also obtained in another control group treated with medium alone (data not shown). On the other hand, the heterospheroid treated with the humanized KM8969 exhibited no positive signals of GM2 (Fig. 5*B*). The results showed that the humanized KM8969 caused the disappearance of GM2-positive SBC-3 cells and prohibited the outgrowth of the rim layers in the heterospheroids that were thought to be SBC-3 cells.

Localization of Humanized KM8969 in Heterospheroids. Immunoperoxidase-stained sections of 7- and 14-day-cultured heterospheroids are shown in Fig. 6. After 7 days of the humanized KM8969 exposure, KM8969 penetrated into the heterospheroid and probed GM2-positive SBC-3 cells that were scattered within the heterospheroid (Fig. 6, *A* and *B*). The result revealed that the binding activity of the humanized KM8969 was preserved even in the heterospheroid. After 14 days, SBC-3 cells migrated from inner cell masses to the surface of the spheroid, and the humanized KM8969 was detected in the thin rim layers of the

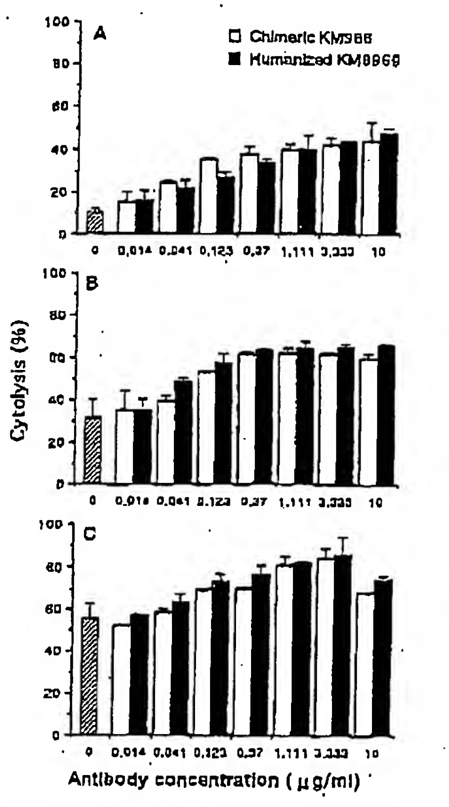

Fig. 3. ADCC of MAbs against human lung cancer SBC-3 cells. ^{51}Cr-labeled SBC-3 cells were incubated at 37°C for 4 h with either chimeric KM966 (*open columns*) or humanized KM8969 (*closed columns*) at the concentrations indicated in the presence of effector cells at E:T ratios of 5:1 (*A*), 10:1 (*B*), or 20:1 (*C*). Effector cells were PBMCs obtained from a healthy donor. After centrifugation, the radioactivity in the supernatants was measured and the percentages of cytolysis were plotted against the concentrations of MAbs. The lysis percentages observed with effector cells alone (*striped columns*) were 9.8 ± 2.0, 31.3 ± 9.3, and 55.1 ± 7.4% for E:T ratios of 5:1, 10:1, and 20:1, respectively.

Table 1 *Effect of humanized KM8969 on human lymphocyte- or monocyte-mediated cytotoxicity against various human lung cancer cell lines*

Target cells	Origin	GM2 expression[a]	MAb (1 μg/ml)	% cytotoxicity[b] (mean ± SD[c])	
				Lymphocyte	Monocyte
RERF-LC-AI	Sq.[d]	2.2		1.7 ± 1.2	0.1 ± 0.7
			KM966	3.1 ± 0.5	0.6 ± 1.0
			KM8969	2.9 ± 0.4	0.8 ± 0.5
SBC-5	SCLC	29.1		5.5 ± 0.9	0.1 ± 1.0
			KM966	20.8 ± 5.6	17.6 ± 1.9
			KM8969	24.3 ± 4.7	23.0 ± 3.4
H69	SCLC	40.3		9.5 ± 6.3	4.9 ± 2.1
			KM966	31.7 ± 3.4	26.0 ± 2.9
			KM8969	35.6 ± 2.9	31.1 ± 3.0
PC-14	Adeno.	45.6		15.0 ± 0.4	1.8 ± 1.1
			KM966	46.8 ± 0.3	30.0 ± 0.8
			KM8969	44.9 ± 1.2	32.5 ± 0.6
PC-13	Large	212.7		11.9 ± 1.4	1.2 ± 0.6
			KM966	57.2 ± 1.7	45.5 ± 1.1
			KM8969	77.7 ± 6.3	47.3 ± 3.4
SBC-3	SCLC	250.7		12.8 ± 2.5	2.2 ± 1.0
			KM966	68.5 ± 4.0	40.9 ± 5.0
			KM8969	78.7 ± 3.3	48.0 ± 2.0

[a] Mean fluorescence intensity analyzed by FACScan using chimeric KM966 (10 μg/ml; Ref. 29).
[b] Lymphocytes or monocytes were incubated for 4 h in medium with or without MAb (1 μg/ml) with ^{51}Cr-labeled cells at E:T ratio of 20:1.
[c] Mean ± SD for triplicate cultures. Data are representative of four separate experiments.
[d] Sq., lung squamous cell carcinoma; Adeno., lung adenocarcinoma; Large, large cell lung cancer.

heterospheroid with heterogeneity (Fig. 6, *C* and *D*). The heterogenic staining pattern of the humanized KM8969 suggested that the existence of GM2-negative SBC-3 cells in the heterospheroid. The sections from two control groups showed no specific staining (data not shown).

Detection of Apoptosis in Heterospheroids. To investigate the mechanism of the growth-inhibitory effect of the humanized KM8969 on the heterospheroid, we undertook experiments to detect apoptosis in the heterospheroid using a method based on *in situ* DNA strand-break labeling. The heterospheroid treated for 14 days with control hIgG exhibited strong positive signals only in the central area of severe necrotic cells caused by the limited supply of oxygen and nutrients (Fig. 7, *A* and *B*). In contrast, the heterospheroid treated for 14 days with the humanized KM8969 exhibited the positive signals of apoptosis distributed heterogeneically at the periphery of the heterospheroid (which resembled the distribution of the humanized KM8969) in addition to the positive signals in the central area (Fig. 7, *C* and *D*). The results suggested that apoptosis of SBC-3 cells induced by the humanized KM8969 contributed to the growth inhibition of the heterospheroid.

In control experiments, the humanized KM8969 affected neither the growth of the heterospheroids composed of normal human dermal fibroblasts and GM2-negative human stomach adenocarcinoma MKN-28 cells nor the growth of SBC-3 cells monolayer cultures (data not shown). These results showed that the growth-inhibitory effect of the humanized KM8969 was strictly dependent on GM2 expression and the multicellular organization of the heterospheroid.

DISCUSSION

We have humanized a murine anti-GM2 MAb in an attempt to improve its potential clinical efficacy by reducing its immunogenicity and by changing the C region to support potent CDC and ADCC. The pharmacokinetic studies of humanized MAbs and murine MAbs in monkeys revealed that the humanization resulted in a prolonged serum half-life and in a substantial reduction in immunogenicity compared with the murine MAbs (44, 45). The antihumanized KM8969 response would be theoretically directed to a conformational epitope formed by the CDR loops, so that the reduction in immune response to the KM8969 in monkeys and humans would be expected in comparison with the chimeric KM966, which has murine V regions including CDRs. There has been no comparative pharmacokinetic studies between the humanized MAbs and their counterpart chimeric MAbs. The actual advantages of the humanized MAbs in pharmacokinetics vary for each MAb and need to be evaluated in clinical studies.

The humanized KM8969 showed a binding affinity and specificity to GM2 similar to the binding affinity and specificity of the chimeric KM966 but showed a 4-fold weaker CDC than that of the chimeric KM966 (Figs. 1 and 2). On the other hand, the humanized KM8969 was slightly more efficient in mediating ADCC than the chimeric KM966 was (Fig. 3). The different activities of CDC and ADCC of the humanized KM8969 may reflect the different binding characteristics between humanized KM8969 and chimeric KM966. In fact, as a result of the humanization of MAbs, different binding characteristics from their parental MAbs have also been reported (46, 47). For example, it is possible that the humanized KM8969 may have kinetic parameters that are different from those of the chimeric KM966. An alternate explanation is that the change of the V region conformation on humanization may affect the interaction between the Fc region and components of the complement or Fc receptor on the effector cell surface. To our knowledge, the humanized KM8969 represents the first humanized anti-GM2 IgG MAb with high binding affinity and specificity. Additional studies of X-ray crystallographic structural analysis and the determination of kinetic parameters of the humanized KM8969 are needed to define the detailed binding mechanisms of anti-GM2 MAbs. These studies are now under way. The results obtained from such studies should be very useful in the humanization of other MAbs.

The humanized KM8969 was able to induce ADCC mediated by both lymphocytes and monocytes against a variety of human lung cancer cell lines in direct proportion to GM2 expression levels on the cell surface (Table 1). Previous studies suggested that the types of effector cells that mediated ADCC varied depending on the nature of the recognized antigen and MAbs (48–50). For example, a chimeric MAb ch14.18 with specificity for GD2 was found to induce ADCC by granulocytes more efficiently than that by NK cells and to have no effect on monocytes (50). Although both lymphocytes and monocytes were able to be effector cells for the KM8969-dependent cytotoxicity, ADCC with lymphocytes seemed to be higher than ADCC with monocytes at an optimal concentration of the KM8969 (1 μg/ml) and an E:T ratio of 20:1. In addition, the humanized KM8969 also induced potent ADCC mediated by both lym-

Table 2 *Effect of humanized KM8969 on human lymphocyte- or monocyte-mediated cytotoxicity against multidrug-resistant or highly metastatic human lung cancer cell lines*

Target cells	Characteristic	MAb (1 μg/ml)	% cytotoxicity[a] (mean ± SD[b])	
			Lymphocyte	Monocyte
SBC-3/ADM	ADM-resistant,		11.1 ± 1.2	0.1 ± 0.5
	P-gp[c] (+), MRP (+)	KM966	50.7 ± 0.1	33.0 ± 2.0
		KM8969	68.8 ± 2.0	27.9 ± 3.7
SBC-3/CDDP	CDDP-resistant		9.6 ± 3.0	1.3 ± 0.6
	P-gp (−), MRP (+)	KM966	71.8 ± 2.9	37.4 ± 1.5
		KM8969	76.4 ± 5.7	39.3 ± 2.2
PC-14-PM4	Highly metastatic to		7.8 ± 0.9	1.3 ± 0.5
	pleural cavity	KM966	39.1 ± 0.8	21.9 ± 0.8
		KM8969	40.0 ± 1.5	25.1 ± 0.9

[a] Lymphocytes or monocytes were incubated for 4 h in medium with or without MAb (1 μg/ml) with ^{51}Cr-labeled cells at E:T ratio of 20:1.
[b] Mean ± SD for triplicate cultures. Data are representative of four separate experiments.
[c] P-gp, P-glycoprotein; MRP, multidrug-resistance-associated protein; +, positive; −, negative.

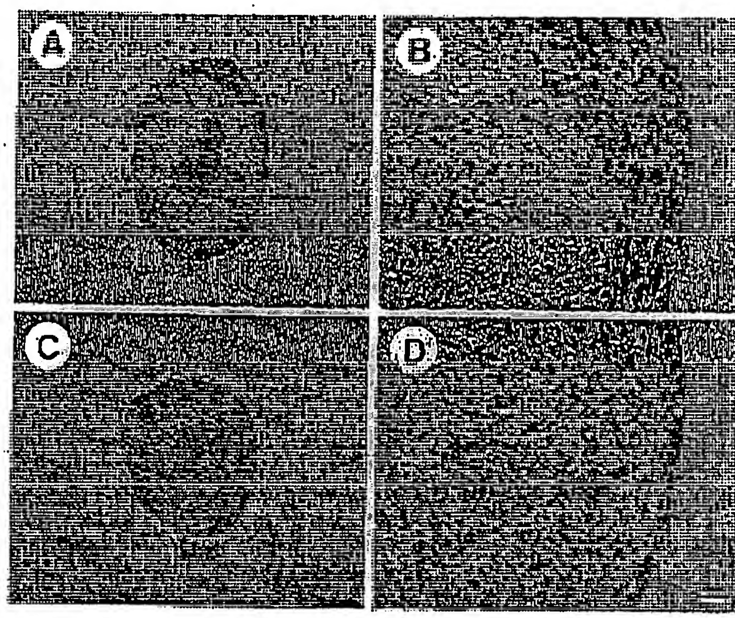

Fig. 4. H&E-stained sections of 14-day-cultured heterospheroids composed of normal human dermal fibroblasts and human lung cancer SBC-3 cells. The heterospheroids were incubated with the antibody from day 2 to 14. The freshly prepared medium containing 20 μg/ml of the antibody was used for each medium exchange at day 2, 4, 6, 8, 10, and 12. The heterospheroids were treated with the control hIgG (*A* and *B*) and with the humanized KM8969 (*C* and *D*). *B* and *D* were high magnification of *A* and *C*, respectively. *Bar*, 100 μm in *A* and *C*; 20 μm in *B* and *D*.

phocytes and monocytes against multidrug-resistant lung cancer cells and highly metastatic lung cancer cells (Table 2). The possibility of the humanized KM8969 being potently useful for overcoming multidrug resistance in cancer cells is very attractive for combination studies with conventional cytotoxic drugs. In addition, potent ADCC against highly metastatic PC-14-PM4 cells of the humanized KM8969 suggested that KM8969 also had the antimetastatic effect *in vivo* similar to the chimeric KM966 (23) and could eradicate the multiple organ micrometastases of human cancers.

Gangliosides are ubiquitous components in the cell surface. Physiological functions of gangliosides have been investigated, and the involvement in the signal transduction of cell growth and the process of cell adhesion has been discussed in relation to oncogenesis and cancer metastasis (30–32). Recently, Iwabuchi *et al.* (35) reported that the GM3-enriched membrane subfraction, termed the glycosphingolipid signaling domain, comprised a structural and functional unit for the initiation of GM3-dependent cell adhesion coupled with signal transduction in mouse melanoma B16 cells. To investigate the role of GM2 in the growth of cancer cells in the form of three-dimensional organization, heterospheroids composed of normal human dermal fibroblasts and GM2-positive human lung cancer SBC-3 cells were cultured in the presence of the anti-GM2 humanized MAb, KM8969.

We found that the growth of the heterospheroids was evidently inhibited on exposure to the humanized KM8969. We noted that the humanized KM8969 induced apoptosis against GM2-positive SBC-3 cells in the heterospheroids, and this effect may play, at least in part, a role in growth inhibition (Fig. 7). The growth-inhibitory effects of MAbs were also reported by other investigators. An anti-p185^{HER2} MAb inhibited the growth of p185^{HER2}-expressing cell lines through weak agonist effects on p185^{HER2} (51). On the other hand, an anti-CD20 MAb showed remarkable growth inhibition against CD20-positive cell lines by apoptosis induction (52). Both MAbs were fully

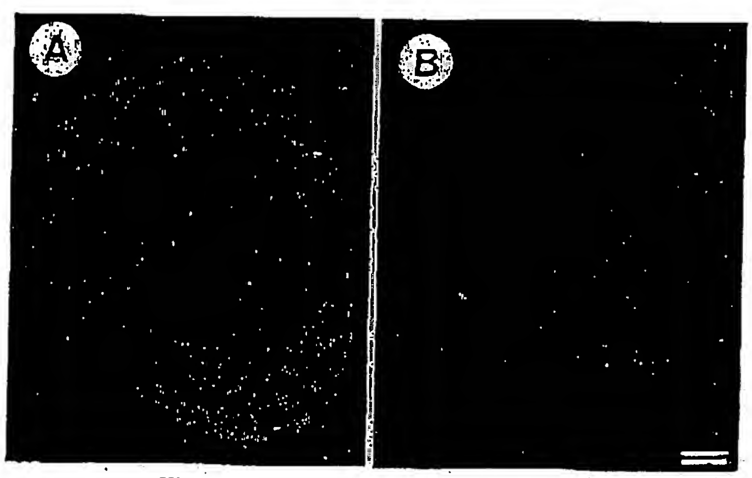

Fig. 5. Detection of GM2 in 14-day-cultured heterospheroids. Heterospheroids were treated with 20 μg/ml antibody on the same schedule as in Fig. 4. GM2 on the heterospheroids was detected using the tagged antibody, KM8969FLAG, followed by biotinylated anti-FLAG and fluorescein-avidin D detection systems. The heterospheroids were treated with the control hIgG (*A*) and with the humanized KM8969 (*B*). *Bar*, 100 μm.

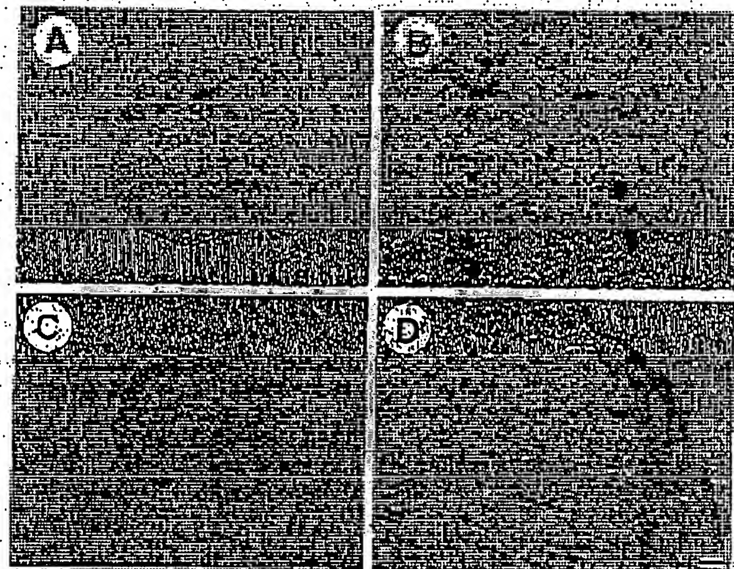

Fig. 6. Detection of the humanized KM8969 in 7- and 14-day-cultured heterospheroids. Heterospheroids were treated with 20 μg/ml antibody on the same schedule as in Fig. 4. The bound humanized KM8969 was detected using horseradish peroxidase-labeled anti-hIgG antibody. *A* and *B*, 7-day-cultured spheroid. *C* and *D*, 14-day-cultured spheroid. *B* and *D* were high magnification of *A* and *C*, respectively. *Bar*: 100 μm in *A* and *C*; 20 μm in *B* and *D*.

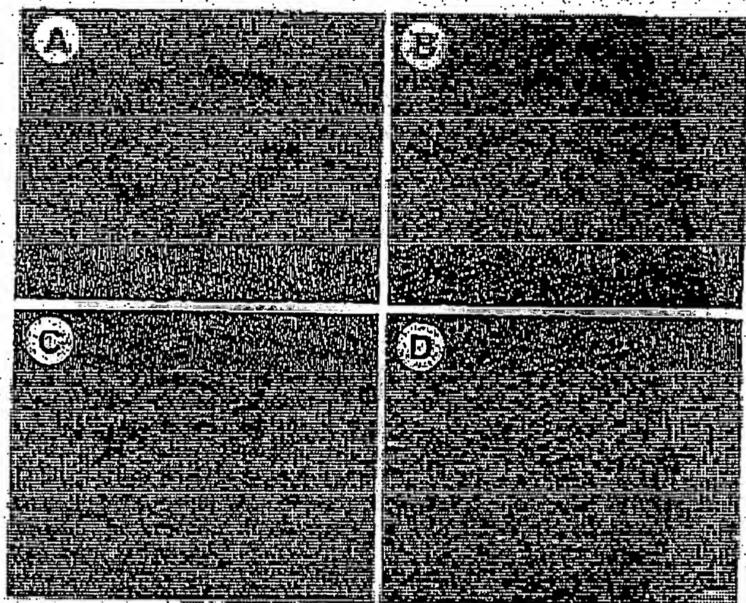

Fig. 7. Detection of apoptosis in 14-day-cultured heterospheroids. Heterospheroids were treated with 20 μg/ml antibody on the same schedule as in Fig. 4. Apoptosis on the heterospheroids was detected using the *In Situ* Cell Death Detection kit, POD. Sections were also counterstained in methyl green. The heterospheroids were treated with the control hIgG (*A* and *B*) and with the humanized KM8969 (*C* and *D*). *B* and *D* were high magnification of *A* and C, respectively. *Bar*: 100 μm in *A* and C; 20 μm in *B* and *D*.

effective in simple monolayer cultures, whereas the humanized KM8969 had no inhibitory effect against monolayer cultures of GM2-positive cell line. The results indicated that the apoptosis of SBC-3 cells was induced by the antibody-capturing by GM2 on the cell surface and by subsequent events in the heterospheroid. Although the detail of the mechanisms of the growth-inhibitory effect of humanized KM8969 remain to be elucidated, the available evidence suggests that GM2 expression on the surface of cancer cells is strongly related to the formation and growth of cancer masses. Additional studies on the cytotoxic effect of the humanized KM8969 using this heterospheroids culture should provide new insights into more effective therapies for GM2-positive human cancers. A very potent cytotoxic agent specific for the growth inhibition of cancer cells could be attained by the cytotoxic functions of the humanized KM8969 because the growth-inhibitory mechanism could enhance the effectiveness of the antibody *in vivo* together with CDC and ADCC.

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